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Phosphonylation of Biomolecules with Inorganic Diphosphonate. II.[1] Phosphonylation of Phosphate Groups on Nucleoside 5′-Monophosphates, Deoxynucleoside 5′-Monophosphates, and Sugar Phosphates

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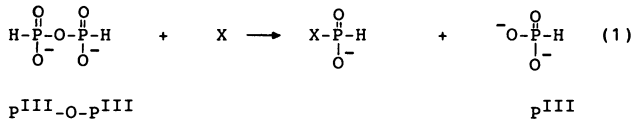
Inorganic diphosphonate phosphonylated predominantly phosphate groups on nucleoside 5′-monophosphates (=NMP's: AMP, GMP, IMP, CMP, and UMP), deoxynucleoside 5′-monophosphates (=dNMP's: dAMP and dTMP), and sugar phosphates (=SP's: Glc1P, Glc6P, and Rib5P) in aqueous solutions under mild conditions (50—70 °C, pH 6). The chemical structures of phosphonylated products were discussed on their ^{31}P NMR spectra and high-performance liquid chromatograms. The main phosphonylated products from NMP's and dNMP's were analogous in structure to nucleoside 5′-diphosphates. The rate of phosphonylation of phosphate groups on NMP's, dNMP's, and SP's varied strongly depending on reaction conditions: pH, reaction temperature, and initial concentration of inorganic diphosphonate. The maximum yields of phosphonylated products were ca. 70% for all NMP's, dNMP's, and SP's under the following conditions: initial concentration of inorganic diphosphonate is 1.5 mol dm^{-3}; each initial concentration of NMP's, dNMP's, or SP's is 0.1 mol dm^{-3}; 70 °C; pH 6.

Blaser and Worms reported[2,3] that disodium inorganic diphosphonate (P^{III}–O–P^{III}), $Na_2P_2H_2O_5$, reacted with several nucleophiles (X) such as orthophosphate and fluoride to form new compounds

(X–P(=O)(O$^-$)–H) and inorganic phosphonate (P^{III}) according to Eq. 1. Hypophosphate,[2,4] diphosphate,[3,5] triphosphate,[5,6] and ammonia[7] were also found to react



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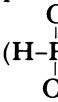
with inorganic diphosphonate to form new compounds. In such reactions a "phosphonyl" group



((H–P(=O)(O$^-$)–) on P^{III}–O–P^{III} is transferred to a nucleophile to form a "phosphonylated" product. We called this type of reaction "phosphonyl transfer reaction" or "phosphonylation" rather than "transanhydrization" proposed by Blaser and Worms,[2,3] because of its similarity to phosphoryl transfer reaction or phosphorylation of biological importance.[8]

Much attention has recently been focused on phosphonylation of biomolecules in the field of nucleic acid syntheses and kinetic research of enzymatic reactions. Successful examples are the application of nucleoside H-phosphonate to automatic syntheses of DNA and RNA,[9,10] and the wide use of phosphonate analogues of biomolecules as a metabolic regulator.[11] On the other hand, no successful attempts with inorganic diphosphonate as a phosphonylating agent have been reported for the synthesis of phosphonylated analogues of "biophosphates" such as nucleotides and sugar phosphates. The main reason is the lack of analytical techniques for simultaneous determination of biophosphates and inorganic phosphonates. We have developed the HPLC system coupled with flow injection analysis for simultaneous determination of biophosphates and inorganic phosphonates.[12–16]

The purpose of our project in this series was to characterize the formation of phosphonylated analogues of biophosphates by means of the HPLC technique[12–16] and ^{31}P NMR spectroscopy. We have already reported[17] that adenosine 5′-monophosphate was phosphonylated by P^{III}–O–P^{III} to give an analogue of adenosine 5′-diphosphate as a main product, with some by-products phosphonylated at the 2′- or 3′-position of the ribose. We also reported in a preliminary paper that a phosphate group on glucose 1-monophosphate was phosphonylated selectively by P^{III}–O–P^{III}.[1] This paper deals mainly with phosphonylation of phosphate groups on nucleoside 5′-monophosphates (=NMP's: AMP, GMP, IMP, CMP, and UMP),[18] deoxynucleoside 5′-monophosphates (=dNMP's: dAMP and dTMP),[18] and sugar phosphates (=SP's: Glc1P, Glc6P, and Rib5P)[18] under various conditions.

Experimental

Chemicals. Disodium salt of AMP from Boehringer-Mannheim, disodium salts of GMP, IMP, CMP, UMP, dAMP, and dTMP from Yamasa Shoyu, and disodium salts

of Glc1P, Glc6P, and Rib5P from Sigma were commercially available.

Disodium inorganic diphosphonate, $Na_2P_2H_2O_5$, was prepared according to the literature[6] with a slight modification. In an Erlenmeyer flask with a glass stopper, 300 ml acetic anhydride, $(CH_3CO)_2O$, was added to a mixture of 18.5 g of phosphonic acid, H_2PHO_3, and 48.6 g of disodium phosphonate pentahydrate, $Na_2PHO_3 \cdot 5H_2O$. The resulting mixture was agitated for 1 h and allowed to be incubated in an ultrasonic cleaner for 3 h at room temperature to give a white precipitate. After the supernatant was removed, the precipitate was suspended in 300 ml acetone, filtered, and washed with acetone in order to remove acetic anhydride and acetic acid. The precipitate was dissolved in 150 ml of water and recrystallized by addition of 210 ml of ethanol. The precipitate was filtered, washed with acetone, and dried at 50 °C to get about 15 g of disodium inorganic diphosphonate. The purity of the disodium inorganic diphosphonate thus obtained was checked by HPLC[12] to be 97—99% (as P).

Reaction of NMP, dNMP, or SP with Inorganic Diphosphonate. Unless otherwise stated, initial concentration of inorganic diphosphonate varied form 0.5 to 1.5 mol dm^{-3}, keeping the initial concentration of NMP's, dNMP's, or SP's at 0.1 mol dm^{-3} constant. The reaction mixture was adjusted to the prescribed pH value (10, 8, 6, or 4) with a sodium hydroxide or hydrochloric acid solution and was allowed to react at 50, 60, 70, and 80 °C. The pH change during the reactions was not controlled strictly.

HPLC Measurement. The HPLC manifold and technique were described in previous papers.[12-16] An HLC-601 system (Toyo Soda) was used as a main pumping system. The separations were performed on a column (100×4.0 mm I.D.) packed with an anion exchanger (TSKgel SAX, 10 μm, Toyo Soda). Flow rate was 1.0 ml min^{-1} and column temperature was kept at 30, 40, or 50 °C. Separated NMP's or dNMP's were detected at 260 nm by use of UV spectrophotometer located at the outlet of the separation column. Phosphate and phosphonate groups on reactants and products were monitored by the on-line coupled flow injection system designed for the differential detection of phosphate and phosphonate groups.[12]

^{31}P NMR Measurement. Pulse FT ^{31}P NMR spectra were recorded at the room temperature by use of JEOL JNM-FX100 (40.25 MHz), VARIAN XL-300 (121 MHz), and JEOL JNM-GX400 (161 MHz) spectrometers. Chemical shifts were measured in comparison with the 85% orthophosphoric acid external standard.

Results and Discussion

Characterization of Phosphonylation by HPLC. An aqueous mixture of AMP and P^{III}–O–P^{III} was incubated at 80 °C. After 3 h incubation, aliquots of the reaction mixture were diluted and analyzed by an HPLC system designed for the simultaneous determination of nucleotides, sugar phosphates, and inorganic phosphonates.[12-16]

Figure 1 shows HPLC profiles for a 100-fold-diluted sample solution. With UV-detection at 260 nm (Fig. 1a), one main product (A) and two by-products (B and C) were observed in addition to a peak for AMP. A small peak for adenosine (Ado), probably as an impurity in AMP, was also recorded.

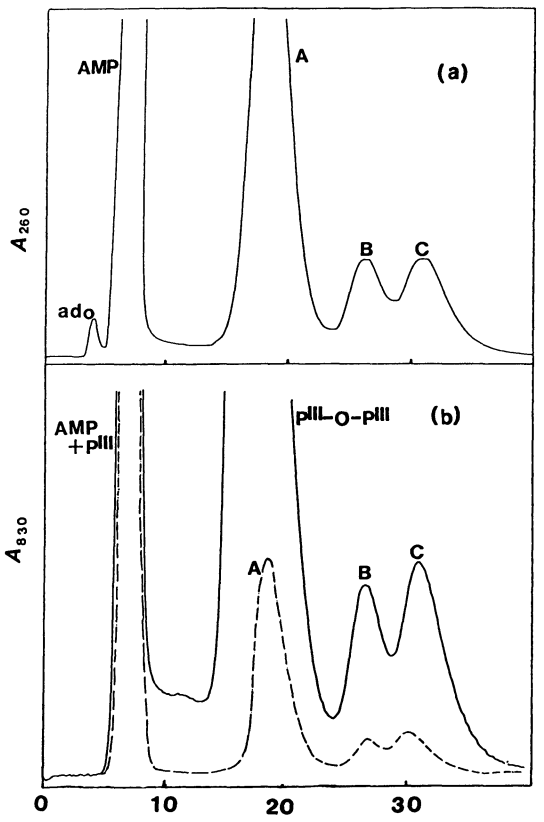


Fig. 1. HPLC profile for AMP (0.15 mol dm^{-3}) phosphonylated by inorganic diphosphonate (0.23 mol dm^{-3}) at 80 °C (pH 6) after 3 h incubation. The reaction mixture was diluted 100-fold. Eluent: 0.12 mol dm^{-3} KCl and 0.1% EDTA 4Na.

The solid lines in Figs. 1b and 2b indicate the sum of the absorbances (830 nm) due to phosphate and phosphonate groups measured in the presence of an oxidizing agent and the broken lines in Figs. 1b and 2b indicate the absorbance (830 nm) due to phosphate group measured in the absence of the oxidizing agent.

The peaks for P^{III} and P^{III}–O–P^{III} in Fig. 1b overlapped with those for AMP and a main product A, respectively. By lowering the sample concentration with 1000-fold dilution, the peaks for P^{III}–O–P^{III}, P^{III}, and A can well be resolved as shown in Fig. 2b. The results in Figs. 1 and 2 indicate that each of species A, B, and C is composed of phosphate, phosphonate, and adenosine, suggesting the formation of derivatives of AMP.

^{31}P NMR Spectra of Phosphonylated Products. No information was available from the HPLC profiles about the chemical structures of the products. To characterize the chemical structures of three phosphonylated products, ^{31}P NMR spectra of the reaction mixture were measured at room temperature on a JEOL JNM-FX100 spectrometer.

A proton-decoupled spectrum of the phosphonylated

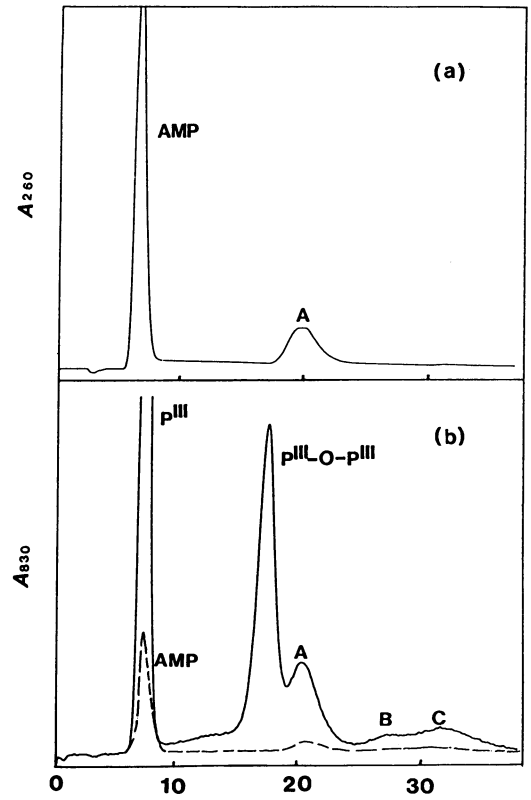


Fig. 2. HPLC profile for AMP (0.15 mol dm^{-3}) phosphonylated by inorganic diphosphonate (0.23 mol dm^{-3}) at 80 °C (pH 6) after 3 h incubation. The reaction mixture was diluted 1000-fold. Eluent: 0.12 mol dm^{-3} KCl and 0.1% EDTA 4Na.

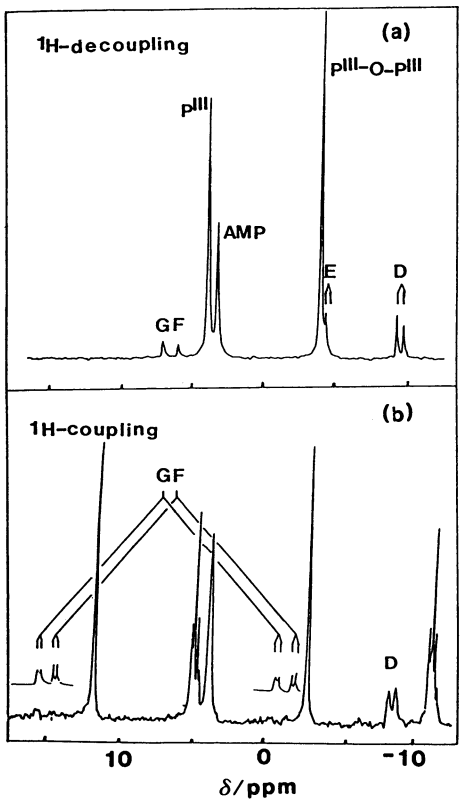


Fig. 3. ^{31}P NMR spectra for AMP (0.1 mol dm^{-3}) phosphonylated by inorganic diphosphonate (0.5 mol dm^{-3}) at 80 °C (pH 6) after 4 h incubation.

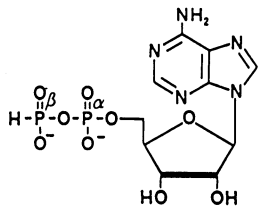


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Chart 1.

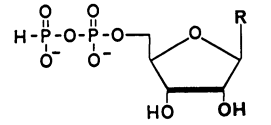
product of AMP (Fig. 3a) shows five singlets and two doublets. Three singlets are assigned to P^{III} (3.93 ppm), AMP (3.30 ppm), and P^{III}-O-P^{III} (−3.93 ppm) on the basis of the chemical shifts for the authentic samples. Two unknown doublets (D and E, −9.55 and −4.00 ppm) and two unknown singlets (G and F, 7.10 and 5.90 ppm) are expected to be the signals for phosphonylated products. One of the signals for the doublet (E) overlapped with that of P^{III}-O-P^{III}. The doublet could be separated from the signal for P^{III}-O-P^{III} by the experiment using a high magnetic field NMR spectrometer (JEOL JNM-GX400). Since the intensities of NMR signals are proportional to phosphorus contents, the main peak (A) in Fig. 1 can be assigned to the doublet (D and E), and the other peaks (B and C) correspond to signals F and G.

The new POP bond is likely to be formed by phosphonylation of AMP because the doublets (D and E) have the same J value of 19.8 Hz and the magnitudes of these J values are approximately equal to the geminal coupling constant ($^2J_{PP}$=21.7 Hz)[19] in adenosine 5′-diphosphate (ADP). The POP bond formation and the intensities of the doublets (D and E) suggest that phosphate group on AMP is predominantly phosphonylated to form product **1** (Chart 1) similar in structure to ADP.

Further splitting of the doublet (E) into a double-doublet is shown in a proton-coupled spectrum (Fig. 3b), while the doublet (D) is not split. The double-doublet (E) is assigned to the phosphorus atom (P_β of **1**) of phosphonyl group (H-P(=O)(O$^-$)-) transferred from P^{III}-O-P^{III} to the phosphate group on AMP because the double-doublet (E) shows a splitting of 665.5 Hz for the hydrogen atom attached directly to a phosphorus atom.[20]

If the doublet (D) can be assigned to the signal of P_α of **1**, the doublet (D) is expected to be further split into double-triplets caused by spin-spin coupling with two 5′-hydrogen atoms. The doublet (D) is not split in Fig. 3b, however, the band widths are broadened

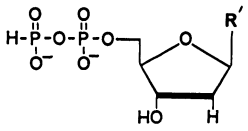
compared with the signal observed in the proton-decoupled spectrum. The magnitude of J value can be estimated to be 4 Hz from band broadening. This value is similar to the vicinal coupling constant ($^3J_{PH}$) in AMP (4.9 Hz).[21] Therefore, the doublet (D) is assigned to the signal of P_α of **1**. Phosphate groups on other NMP's (Chart 2), dNMP's (Chart 3), and SP's (Chart 4) were also confirmed to be phosphonylated predominantly. Table 1 lists NMR parameters for phosphonylated products.



2~5

2: R=guanine
3: R=hypoxanthine
4: R=uracil
5: R=cytosine

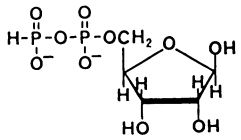
Chart 2.



6~7

6: R′=adenine
7: R′=thymine

Chart 3.



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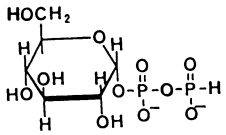

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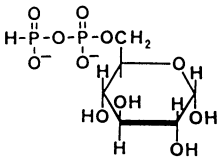

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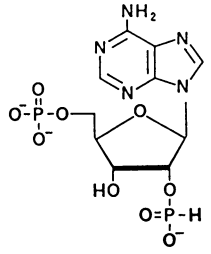
Chart 4.

The signals corresponding to G and F in Fig. 3a cannot be observed in a proton-coupled spectrum as shown in Fig. 3b, owing to a complex splitting and a low yield. Signals G and F are assigned to compounds **11** and **12** (Chart 5) produced by phosphonylation of



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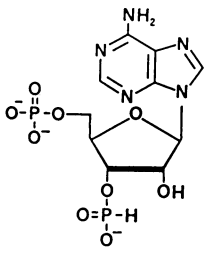

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Chart 5.

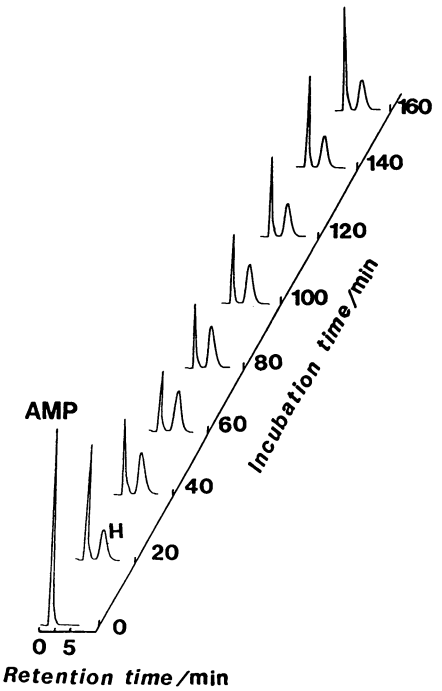

Fig. 4. Kinetic HPLC profile for phosphonylation of AMP (0.1 mol dm^{-3}) with inorganic diphosphonate (1.5 mol dm^{-3}) at 60 °C and pH 6. Eluent: 0.20 mol dm^{-3} KCl and 0.1% EDTA 4Na.

Table 1. ^{31}P NMR Parameters of Phosphonylated Products

Product	$\delta(P_\alpha)$	$\delta(P_\beta)$	$^1J_{P_\beta H}$/Hz	$^2J_{P_\alpha P_\beta}$/Hz	$^3J_{P_\alpha H}$/Hz
1	−9.51	−4.03	667	19.8	4[a]
2	−7.18	−1.30	665	18.8	4[a]
3	−9.35	−4.12	665	19.8	4[a]
4	−9.64	−4.19	666	19.8	4[a]
5	−9.67	−4.19	665	19.1	4[a]
6	−9.53	−4.11	665	19.8	4[a]
7	−10.4	−4.87	665	19.8	4[a]
8	−9.63	−4.29	663	19.6	6.5
9	−11.3	−4.18	666	19.5	7.26
10	−9.45	−4.19	664	19.8	5.81

a) Estimated from the band broadening.

2′- or 3′-OH group, because the double-doublets (+5.9 and +7.1 ppm, $^1J_{PH}$=650 Hz and $^3J_{PH}$=10 Hz each) are observed in a proton-coupled spectrum by other experiments under alkaline conditions (pH 10). Phosphonylation of AMP in an alkaline solution gave **11** and **12** predominantly, but in a neutral solution, **11** and **12** were formed in small amounts (<2%). Phosphonylation of OH groups will be described in detail in a subsequent paper of this series.

Kinetic Profiles for Phosphonylation. Figures 4 and 5 show kinetic HPLC profiles for phosphonylation of AMP and Rib5P. The reaction mixture (P^{III}-O-P^{III}; 1.5 mol dm^{-3}, AMP or Rib5P; 0.1 mol dm^{-3}) was incubated at 60 °C in the pH range from 6 (0 min) to 5.5 (after 100 min) without any buffer solution. Aliquots (0.1 ml) of mixed solutions were withdrawn at 10 or 20 min intervals and diluted with 100 ml of distilled water. An aliquot (0.1 ml) of diluted solutions was introduced into an HPLC system designed for determination of nucleotides and both phosphate and phosphonate groups on reactants and products.[12-16] In the reaction of NMP's and dNMP's, the reactant and the product were detected simultaneously at 260 nm as shown in Fig. 4. On the other hand, phosphate groups were detected in the reaction of SP's to determine SP's and a phosphonylated product simultaneously as shown in Fig. 5, since SP's and their products **8**, **9**, and **10** have no UV-absorption.

The HPLC profiles at time 0 in Figs. 4 and 5 showed the peaks of only AMP and Rib5P. The elution peaks (H and I) appearing after 10—20 min represent the peaks of the phosphonylated products **1** and **8**. The peak areas of the products increased with time at the expense of AMP and Rib5P. The yields of

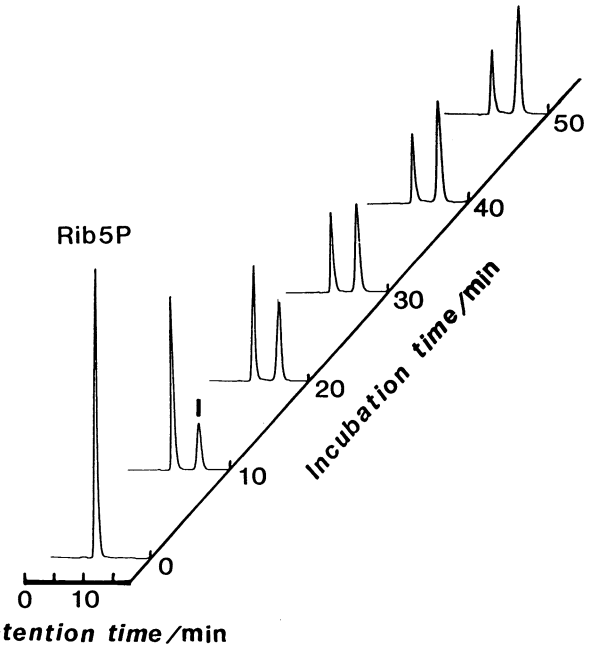

Fig. 5. Kinetic HPLC profile for phosphonylation of Rib5P (0.1 mol dm^{-3}) with inorganic diphosphonate (1.5 mol dm^{-3}) at 60 °C and pH 6. Eluent: 0.20 mol dm^{-3} KCl and 0.1% EDTA 4Na.

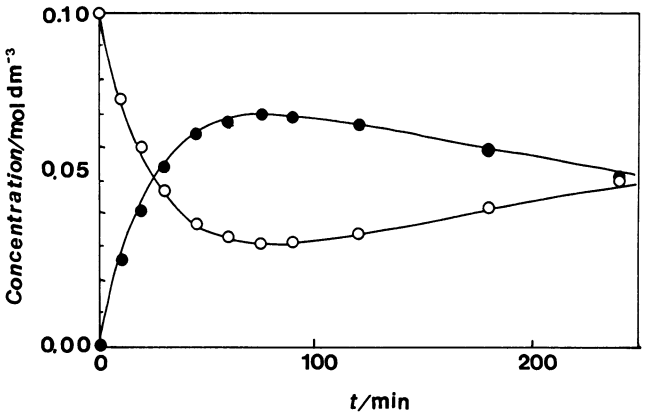

Fig. 6. Time dependence of the reaction components of phosphonylation for AMP with inorganic diphosphonate. Plot of the observed molar concentrations of AMP and product **1** vs. time/min in aqueous solution containing initially 0.1 mol dm^{-3} AMP and 1.0 mol dm^{-3} P^{III}-O-P^{III} at 60 °C and pH 6. (○): AMP, (●): product **1**. Eluent: 0.20 mol dm^{-3} KCl and 0.1% EDTA 4Na.

Table 2. Phosphonylation of AMP with P^{III}-O-P^{III}

Reaction condition					Yield/%
Molarity/mol dm^{-3}		pH	Temp/°C	Time/min	
P^{III}-O-P^{III}	AMP				
0.5	0.1	6	50	480	41
			60	300	43
			70	180	49
1.0	0.1	4	60	60	18
		6	50	300	59
			60	120	60
			70	60	62
		8	60	120	53
		10	60	0—120	0
1.5	0.1	6	50	180	69
			60	75	70
			70	30	69

the products reached a maximum after 50—60 min, and then gradually disappeared.

Effects of pH and Temperature on the Phosphonylation of NMP's. Figure 6 illustrates the time-dependent utilization of AMP and formation of product **1**. With the progress of the reaction, the amount of **1** increased gradually to a maximum (about 70% after 60 min), and then gradually decreased. Table 2 lists maximum yields and reaction times required to reach maximum yields under various reaction conditions.

The pH effect on the maximum yield was examined in the reaction of AMP at 60 °C and 1.0 mol dm^{-3} of initial concentration for P^{III}–O–P^{III}. In the pH range studied here, products were obtained in higher yields at neutral pH region, compared to a lower yield in both acidic and alkaline pH regions. The optimal pH was confirmed to be 6, because the reaction at pH ca. 6 gave a maximum yield, and the mixed solutions of P^{III}–O–P^{III} and NMP's, dNMP's, or SP's were kept at pH ca. 6 without any buffer solutions.

The maximum yield increased with increasing initial concentration of P^{III}–O–P^{III}: 50% at 0.5 mol dm^{-3}, 60% at 1.0 mol dm^{-3}, and 70% at 1.5 mol dm^{-3}. The increase in reaction temperature decreased the time required to reach a maximum yield, but did not contribute to an increase in a maximum yield. The optimal conditions for phosphonylation of AMP with P^{III}–O–P^{III} were found to be initial concentration of P^{III}–O–P^{III} 1.5 mol dm^{-3}, pH 6, and 60 °C.

Table 3. Yields/% of Phosphonylated Products of NMP's[a]

NMP[b]	[P^{III}–O–P^{III}]/mol dm^{-3}	Time/min		
		30	60	120
GMP	1.0	26	42	56
IMP	1.0	26	43	59
	1.5	43	61	66
UMP	1.0	25	42	57
	1.5	44	61	66
CMP	1.0	29	46	62
	1.5	50	68	73

a) Reaction temperature: 60 °C; pH: 6. b) Initial concentration of NMP is 0.1 mol dm^{-3}.

To examine effects of bases of NMP's on maximum yields, the maximum yields of GMP, IMP, UMP, and CMP were compiled in Table 3. The maximum yields and reaction times were almost the same as that obtained for the reaction of AMP. Therefore, we concluded that differences in bases on NMP's did not change the reactivity of NMP's even in the purine and pyrimidine nucleotides.

Effects of pH and Temperature on the Phosphonylation of dNMP's and SP's. Both dNMP's and SP's were also phosphonylated with P^{III}–O–P^{III} under various conditions and the maximum yields were listed in Tables 4 and 5.

dNMP was phosphonylated after 2 h in the yield of 59% at pH 6 and 60 °C when the initial concentration of P^{III}–O–P^{III} was 1.0 mol dm^{-3}. The yield increased and the reaction time decreased with an increase in initial concentration of P^{III}–O–P^{III} as well as in the reaction of NMP. In the reaction of dAMP the yield of **7** was found to be 57 and 70% at 1.0 and 1.5 mol dm^{-3} of P^{III}–O–P^{III}, respectively. The optimum conditions for the phosphonylation of dNMP's are at 1.5 mol dm^{-3} of P^{III}–O–P^{III}, pH 6, and 60 °C.

In the reaction of Rib5P, the yield of **8** at pH 6 was higher than that at pH 4 and 8 under the conditions of 1.0 mol dm^{-3} of P^{III}–O–P^{III} and 60 °C. At 1.5 mol dm^{-3} of P^{III}–O–P^{III}, Rib5P was phosphonylated in the yield of 70% at pH 6 and 60 °C, while the yields of **8** were 43 and 59% when the initial concentrations of P^{III}–O–P^{III} were 0.5 and 1.0 mol dm^{-3}, respectively. Initial concentration affected yields strongly, but reaction temperature did not contribute to changes in the yield in spite of decreasing reaction time. At pH 6 and 60 °C, other SP's were also phosphonylated by P^{III}–O–P^{III}. About 70% of Glc1P or Glc6P was phosphonylated after 1 h at 1.5 mol dm^{-3} of P^{III}–O–P^{III}. The optimal conditions for the phosphonylation of SP's are at 1.5 mol dm^{-3} of P^{III}–O–P^{III}, pH 6, and 60 °C as well as in the reaction of NMP's and dNMP's.

The results obtained in the reaction of dNMP's or SP's showed that the 2′-OH and bases on NMP's affected slightly the reactivity of phosphate groups on NMP's, dNMP's, and SP's.

Table 4. Phosphonylation of dNMP's with P^{III}–O–P^{III}

Reaction condition					Yield/%
Molarity/mol dm^{-3}		pH	Temp/°C	Time/min	
P^{III}–O–P^{III}	dTMP				
1.0	0.1	4	60	60	15
		6	60	120	59
		8	60	120	51
1.5	0.1	6	60	120	66
P^{III}–O–P^{III}	dAMP				
1.0	0.1	6	60	120	57
1.5	0.1	6	60	60	68

Table 5. Phosphonylation of SP's with P^{III}–O–P^{III}

Reaction condition					Yield/%
Molarity/mol dm^{-3}		pH	Temp/°C	Time/min	
P^{III}–O–P^{III}	Rib5P				
0.5	0.1	6	50	420	34
			60	270	43
		8	70	180	42
1.0	0.1	4	60	100	31
		6	50	240	61
			60	120	59
			70	60	57
		8	60	60	38
1.5	0.1	6	50	120	69
			60	60	70
			70	30	70
P^{III}–O–P^{III}	Glc1P				
1.0	0.1	6	60	120	60
1.5	0.1	6	60	60	70
P^{III}–O–P^{III}	Glc6P				
1.0	0.1	6	60	120	68
1.5	0.1	6	60	60	68

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18) Abbreviations: NMP, nucleoside 5′-monophosphate; AMP, GMP, IMP, CMP, and UMP, adenosine, guanosine, inosine, cytidine, and uridine 5′-monophosphate; dNMP, deoxy-nucleoside 5′-monophosphate; dAMP and dTMP, deoxy-adenosine and thymidine 5′-monophosphate; SP, sugar phosphate; Glc1P, Glc6P, and Rib5P, glucose 1-monophosphate, glucose 6-monophosphate, and ribose 5-monophosphate.

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